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Hormonally Derived Sex Pheromones in Goldfish: A Model for Understanding the Evolution of Sex Pheromone Systems in Fish

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Abstract. It is now well established that female goldfish release unmodified and metabolized sex hormones to the water and that some of these compounds function as potent sex pheromones detected by the male's olfactory sense. In goldfish, both olfactory pheromonal receptors and their corresponding hormonal receptors appear to be transmembrane-domain receptors coupled with G proteins. Recent studies of other teleost fish indicate that fish commonly use 'hormonal-pheromones.' Taken together, these data suggest that fish pheromone systems may have evolved as a consequence of a chance expression of hormone receptor molecules on olfactory receptor cells. Isolation and identification of olfactory and hormonal receptors may be the next step in resolving this question.

Introduction

Pheromones have been defined as "substances that are excreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example a definite behaviour or developmental process" (Karlson and Luscher, 1959). Living in an aquatic environment generally devoid of visual cues but rich in dissolved compounds, fish have evolved highly developed chemosensory and pheromonal signalling systems. Where studied, these pheromonal systems have been found to play fundamental roles promoting reproductive synchrony, and there are many examples of the odor of sexually mature fish stimulating either a physiological process or a behavior (Liley, 1982; Stacey and Sorensen, 1991). However, until our recent serendipitous discovery that ovulatory female goldfish, *Carassius auratus*, release the steroidal sex hormone 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) to the water where it then functions as a pre-ovulatory sex pheromone (Dulka *et al.*, 1987), not a single fish sex pheromone had been clearly identified. Although our discovery that an unmodified hormone functioning as a pheromone seemed surprising at the time, Kittredge *et al.* (1971) had predicted almost 20 years earlier that aquatic organisms commonly use hormonal compounds as pheromones. Kittredge *et al.* (1971) based this prediction on the apparent evolutionary ease with which such a system might evolve: hormonal cues are both naturally released and inherently meaningful, and their recognition might require only one step (mutation), the chance expression of hormonal receptors on chemosensory receptor cells.

Recent investigations indicate that fish may commonly use 'hormonal-pheromones.' In addition to 17,20βP, the goldfish uses at least one other major class of hormonal compounds, the F prostaglandins (PGFs), as sex pheromones (Sorensen *et al.*, 1988). Additionally, at least a dozen examples of other fish species whose olfactory systems are acutely and specifically sensitive to hormonal compounds are now known (Stacey and Sorensen, 1991; Sorensen *et al.*, 1992). These findings in fish are not the first to document a parallel between internal and external chemical signalling systems. Almost 40 years ago, Haldane (1954) noted that acetylcholine and epinephrine altered protozoan swimming patterns and hypothesized that the internal signalling systems found in metazoa may have evolved from external (pheromonal) systems used by their unicellular ancestors. Today at least a dozen neuroactive substances from vertebrates are known to stimulate invertebrate chemosensory responses. Reviewing the biochemical mechanisms underlying the internal and chemosensory receptor systems for these substances, Carr *et al.* (1989) conclude that enough similarities exist to 'provide strong support for Haldane's (1954) hypothesis.' Do similarities also exist between hormonal and pheromonal

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receptor systems in th[illegible] And if they do, is Haldane's (1954) hypot[illegible]ptor 'internalization,' or Kittredge *et al.*'[illegible]esis of receptor 'externalization,' the b[illegible]ion? This paper will address these questi[illegible] goldfish hormonal-pheromone system.

A Brief Overview of the Goldfish Hormonal-Pheromone System

Because we have recently published several reviews on the goldfish hormonal-pheromone system (Stacey and Sorensen, 1991; Sorensen *et al.*, 1991; Stacey, 1991; Sorensen, 1992), I will only review the most pertinent elements here. Briefly, female goldfish ovulate in the early morning when light levels are low and, like other oviparous teleosts, become sexually receptive at the time of ovulation (Stacey, 1987). Because ovulated eggs remain viable for but a few hours within the female, females must, and generally do, spawn (release their eggs) within a few hours; male-female reproductive physiology and behavior must be tightly synchronized. This synchrony appears mediated by at least two hormonal-pheromones released in a sequential manner by ovulatory females. Although there is evidence for a third steroidal pheromone resembling androstenedione which has inhibitory actions on male gonadotropin (GtH) release (Stacey, 1991; Sorensen *et al.*, 1991), we will not discuss it here because it is still poorly understood.

In goldfish, as in the vast majority of externally fertilizing fish, oocyte final maturation (resumption of meiosis) is stimulated by a surge in GtH release which in turn stimulates synthesis of the steroidal maturational hormone 17,20βP by ovarian follicles (Stacey, 1987). Circulating levels of immunoreactive-17,20βP increase dramatically during the 10–12 h period preceding ovulation in goldfish and then collapse at the time of ovulation, which coincides with spawning (Stacey *et al.*, 1989). Circulating 17,20βP is rapidly cleared to the water (by unknown means) where it then functions as a potent odorant with pheromonal actions (Dulka *et al.*, 1987). Although much of the blood-borne 17,20βP is released unmodified as a free steroid, considerable quantities are conjugated with either glucuronic acid (17,20βP-G) or sulfate (17,20βP-S), and this mixture is accompanied by a variety of non-pheromonally active steroids (Sorensen *et al.*, 1991); it is unclear whether 17,20βP release reflects a specialized female signaling system. As assayed by electro-olfactogram (EOG) recording from the olfactory epithelium, and confirmed by whole-animal bioassays, the goldfish olfactory system is acutely sensitive to 17,20βP and 17,20βP-S, but not 17,20βP-G (Sorensen *et al.*, 1991). Although 17,20βP and 17,20βP-S appear to be detected by different receptor mechanisms, both stimulate male GtH release (Sorensen *et al.*, 1991), and evoke increased sperm production by the time of spawning (Dulka *et al.*, 1987). Because neither steroid appears to have immediate dramatic influences on male behavior, this pre-ovulatory pheromone is considered a 'primer' (Sorensen *et al.*, 1989).

At the time of ovulation (when final maturation is complete) female goldfish become sexually receptive and release another pheromone which is derived from circulating prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). In goldfish, and presumably many other fish, $PGF_{2\alpha}$, or a compound closely resembling it, appears to have both a paracrine function modulating ovulation (Stacey and Goetz, 1982), and a hormonal function stimulating female spawning behavior (Stacey, 1987). Evidence for these roles is extensive but somewhat indirect. As in most vertebrates, $PGF_{2\alpha}$ stimulates follicular rupture in goldfish oocytes both *in vitro* and *in vivo* (see Goetz *et al.*, 1991), and $PGF_{2\alpha}$ is measurable in cultures of goldfish oocytes (Goetz, 1991). Additionally, exposure to indomethacin, a prostaglandin synthetase inhibitor, blocks ovulation both *in vitro* and *in vivo* (Stacey and Goetz, 1982). Evidence that $PGF_{2\alpha}$ functions as a behavioral hormone stimulating female sexual behavioral receptivity comes from the discovery that interperitoneal injections of $PGF_{2\alpha}$ elicit apparently normal sexual receptivity in non-ovulated females within minutes (Stacey, 1976). Because $PGF_{2\alpha}$ injection is most effective if injected directly into the cranium (Stacey and Peter, 1979), direct actions on the brain are strongly suspected. These findings receive support from the observation that PGF titers increase dramatically in the blood of recently ovulated goldfish (Bouffard, 1979) and the fact that females exhibit sexual behavior only when holding ovulated eggs (Stacey and Goetz, 1982).

Recent evidence now suggests that circulating $PGF_{2\alpha}$ associated with the presence of ovulated eggs is rapidly metabolized and cleared to the water where it then functions as a pheromone stimulating male sexual behavior. PGFs may thus synchronize ovulation, female sexual behavior, and male arousal. Indications of this possibility first arose with the observation that non-ovulated goldfish injected with $PGF_{2\alpha}$ release an odor which elicits male reproductive behavior (Sorensen *et al.*, 1986). Recently we discovered that as measured by EOG recording, the goldfish olfactory system is acutely and specifically sensitive to waterborne $PGF_{2\alpha}$ and a mammalian metabolite 15-keto-prostaglandin $F_{2\alpha}$ (15k-$PGF_{2\alpha}$) (Sorensen *et al.*, 1988). The relevance of this was confirmed by the finding that males exposed to waterborne PGFs become sexually active, and that ovulated females release immunoreactive PGFs to the water (Sorensen *et al.*, 1988). Most recently, we have injected goldfish with radiolabeled $PGF_{2\alpha}$ and established that they release substantial quantities of at least three unknown metabolites of $PGF_{2\alpha}$ but not 15K-$PGF_{2\alpha}$ suggesting that the pheromone is a mixture of several novel PGF metabolites (unpub.).

Comparing Hormonal and Pheromonal Functions of 17,20βP and PGFs in Goldfish

Although not a single vertebrate olfactory receptor molecule has been identified, a preponderance of evidence suggests that olfactory receptors are members of a superfamily of surface receptors that cross the cellular membrane seven times and are associated with guanine nucleotide-binding proteins (G proteins) (Anholt *et al.*, 1991). The most direct evidence for this possibility comes from Buck and Axel (1991) who constructed oligonucleotide probes to demonstrate the existence of an extremely large family of genes encoding for G protein-linked transmembrane proteins expressed exclusively in olfactory tissue isolated from rats. Although this superfamily of transmembrane proteins appears to meet all of the criteria of olfactory receptors, direct proof is still lacking. Buck and Axel (1991) note that 'the detection of odors at the surface in the periphery is therefore likely to involve signaling mechanisms shared by other hormone or neurotransmitter systems.' They also note that 'members of this family of olfactory proteins are conserved in lower vertebrates and . . . presumably extended over evolutionary time.' Here I examine the goldfish hormonal-pheromone system to determine whether hormonal and pheromonal receptor mechanisms share the same biochemical/biophysical mechanisms, keeping in mind that if they do, this might reflect common evolutionary origins.

Ligand-binding studies using goldfish olfactory epithelium have clearly established that binding activity for 17,20βP is associated with neural membranes (Rosenblum *et al.*, 1991)—as is the case for all vertebrate olfactory binding activity studied to date (Anholt *et al.*, 1991), including that for feeding attractants (amino acids) in fish (see Bruch and Rulli, 1988). Ligand-binding studies have not been conducted for PGFs, but electrical responses elicited by PGFs are extremely similar to those elicited by amino acids and 17,20βP—there is every reason to suspect that they are also associated with the cell membrane. Signal transduction mechanisms associated with 17,20βP and PGF olfactory responsiveness have not yet been studied, but signal transduction mechanisms have been extensively investigated in fish in association with the olfactory responsiveness of catfish (*Ictalurus punctatus*) to amino acids. The mechanisms described are similar to those found in higher vertebrates (see Anholt *et al.*, 1991). Briefly, the catfish studies indicate that amino acid olfactory receptors are coupled to G protein(s) which may activate either cAMP-gated cation channels (Bruch and Teeter, 1989), or inositol triphosphate (IP3)-gated calcium channels (Restrepo *et al.*, 1990). Although fish olfactory G protein(s) have not been identified, it is fascinating that a mammalian olfactory G protein has been (G_{olf}), and it has a high degree of homology (88%) with conventional $G_{s\alpha}$ (Jones and Reed, 1989).

It is striking that the hormonal actions of both 17,20βP and $PGF_{2\alpha}$ also appear to be mediated by membrane receptors associated with G proteins. 17,20βP appears to exert its actions on the fish oocyte via a membrane receptor, a scenario resembling the actions of progesterone on the amphibian oocyte (see Jalabert *et al.*, 1991). Direct evidence for this possibility in the goldfish comes from the observation that 17,20βP stimulates final maturation when applied to the oocyte surface, but is ineffective if injected directly into the oocyte cytoplasm (data reported by Nagahama, 1987). Indirect support comes from other species of fish in which ligand-binding studies have demonstrated a high level of specific binding activity for 17,20βP-related steroids to oocyte membranes (see Patino and Thomas, 1990). Additionally, although no direct evidence of G protein involvement in 17,20βP-induced final maturation has been reported, it is quite clear that adenylate cyclase and cAMP are involved because administration of either cAMP or forskolin to oocytes blocks maturation. The latter finding is thought to indicate that 17,20βP exerts its actions by reducing cAMP, thereby reducing the phosphorylation of an inhibitory substrate by cAMP-dependent protein kinase (see Jalabert *et al.*, 1991). These findings suggest that the 17,20βP membrane receptor might be coupled to an inhibitory G protein. Unfortunately, $PGF_{2\alpha}$ binding has not been studied in either the fish ovary or brain. However, where studied in mammals, $PGF_{2\alpha}$ binding activity has been found associated with cell membranes in both the ovary (Rao, 1976) and brain (Watanabe *et al.*, 1985). Additionally, in goldfish there is strong evidence that G proteins are associated with ovulation—orthovanadate, a G-protein stimulator, evokes both IP_3 production and ovulation (Ranjan and Goetz, 1990). Signal transduction mechanisms associated with the behavioral effects of $PGF_{2\alpha}$ have not been studied.

Existing evidence suggests that both pheromonal (olfactory) and hormonal receptors for 17,20βP and PGF in goldfish are transmembranal and linked to G proteins—supporting the possibility that these systems might be evolutionarily related. But what about the relative specificities and sensitivities of goldfish olfactory and hormonal receptors? As measured by EOG recording (Sorensen *et al.*, 1990) and radio-receptor assay (Rosenblum *et al.*, 1991), olfactory structure-activity relationships for 17,20βP binding appear quite similar to those described by hormonal studies assaying the effects of steroids on final maturation (Jalabert, 1976). However, although not measured, it seems quite unlikely that the pheromonally active metabolite 17,20βP-S has hormonal activity. A similar scenario exists for the PGFs—the pheromonally active metabolite 15K-$PGF_{2\alpha}$ has little ability to stimulate female sexual behavior (Sorensen *et al.*, 1987). Do these differences in olfactory and hormonal receptor binding characteristics negate the possibility that these systems are related? Not necessarily, at least if Kittredge *et al.*'s

(1971) hypothesis is c[illegible] seems quite reasonable that olfactory hormonal p[illegible] receptors should quickly diversify in acco[illegible] speciation events and the pressures for p[illegible] ecies-isolation mechanisms that must accom[illegible] Hormone metabolism and release mechanisms [illegible] also be expected to exhibit diversification. The existence of both 17,20βP and 17,20βP-S binding activity in the goldfish epithelium may be an example of pheromonal-evolutionary divergence.

A final consideration is whether it is conceivable that a transmembranal hormonal receptor expressed in an olfactory membrane could interact functionally with olfactory G proteins to transduce a response. Although this experiment has not been performed, receptor proteins have been transferred to foreign cell membranes and remained functional (Citri and Schramm, 1980). Furthermore, G_{olf} is apparently similar enough to $G_{s\alpha}$ that it will stimulate adenylate cyclase activity when expressed in lymphoma cells (Jones and Reed, 1989). In summary, what little we know about pheromonal receptor mechanisms and their hormonal counterparts in goldfish suggest they are strikingly similar, perhaps similar enough that they be evolutionarily related.

Hormonal-Pheromones in Other Species of Fish: The Question of Species-Specificity and Its Relevance to the Evolution of Hormonal-Pheromones

It is important to understand the diversity (species-specificity) of hormonal-pheromone systems because it reflects on how these systems may have evolved. This is especially the case because fish hormonal systems are thought to be highly similar ('conserved'); 17,20βP, or a very closely related steroid, has been found to serve as the maturational hormone in all fish examined to date (Scott and Canario, 1987). Although paracrine and hormonal $PGF_{2\alpha}$ function is less well understood, evidence suggests that it too is used by many fishes (Stacey, 1987; Goetz *et al.*, 1991). In contrast, surveys of EOG sensitivity of various fishes increasingly indicate that hormonal-pheromones might be species-specific (Sorensen *et al.*, 1992). For instance, EOG recording has now been used to describe the olfactory sensitivities of at least half a dozen members of the family Cyprinidae to almost 20 hormonal compounds. Of these species, only the goldfish and the closely related common carp, *Cyprinus carpio*, detect either 17,20βP or 17,20βP-S; the rest detect 17,20βP-G. Perhaps even more striking, the olfactory sensitivity of three species from the family Catastomidae, a family in the same order as the Cyprinidae, have now been tested and no sensitivity to any sex steroids has been measured (Sorensen *et al.*, 1992). Similarly, although all members of the Cyprinidae and Catastomidae tested respond to PGFs, there are clear differences in relative responsiveness. In spite of the fact that these data must be interpreted cautiously because of the small number of species and individuals tested, uncertainties about the meaning of EOG recording, and our poor understanding of hormone metabolism and excretion in fish—it seems safe to conclude that fish pheromone systems are more diverse than the hormone systems from which they are metabolically derived.

As previously mentioned, species-specificity in hormonal-pheromone system function may not be surprising, but when associated with a highly conserved hormonal system, as appears to be the case in fish, it strongly suggests that if one of these systems is evolutionarily derived from the other, the hormonal system is the likely predecessor. It is a simple matter to imagine how a common hormonal-pheromone system might come to exhibit evolutionary radiation, but nearly impossible to imagine how and why a highly conserved hormone system could evolve from a pre-existent, diverse pheromone system. An alternative possibility that I have not yet considered is that hormonal and pheromonal receptor systems may be independently derived from common, extremely ancient, and unknown chemoreceptor systems. However, I presently know of no data that support this possibility. In conclusion, existing evidence from the goldfish hormonal-pheromone system, and fish in general, supports Kittredge *et al.*'s (1971) hypothesis that pheromonal signaling systems may reflect an externalization of internal hormonal signalling systems. Definitive proof of this hypothesis awaits receptor identification, a project I hope this treatise will encourage.

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